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Catabolite repression of the *citST* two-component system in *Bacillus subtilis*

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Abstract

In *Bacillus subtilis*, expression of the citrate transporter CitM is under strict control. Transcription of the *citM* gene is induced by citrate in the medium mediated by the CitS–CitT two-component system and repressed by rapidly degraded carbon sources mediated by carbon catabolite repression (CCR). In this study, we demonstrate that *citST* genes are part of a bicistronic operon. The promoter region was localized in a stretch of 58 base pairs upstream of the *citS* gene by deletion experiments. Transcription of the operon was repressed in the presence of glucose by the general transcription factor CcpA. A distal consensus *cre* site in the *citS*-coding sequence was implicated in the mechanism of repression. Furthermore, this repression was relieved in *Bacillus subtilis* mutants deficient in CcpA or Hpr/Crh, components essential to CCR. Thus, we demonstrate that CCR represses the expression of the *citST* operon, which is responsible for the induction of *citM*, through the *cre* site located 1326 bp from transcriptional start site of *citST*.

Introduction

Bacillus subtilis is a metabolically versatile Gram-positive soil bacterium. The microorganism has a complete set of enzymes for the tricarboxylic acid (TCA) cycle and can grow aerobically using most of the TCA cycle intermediates (malate, fumarate, succinate and citrate) as a sole carbon source (Sonenshein, 2002). When preferred carbon sources such as glucose are not present in the environment a hierarchical utilization of TCA cycle intermediates and other compounds allows this bacterium to survive in nature. The selection of the energetically most convenient nutrient is mediated by a complex, intertwined signal transduction network that controls the carbon and nitrogen utilization pathways. As part of this network, two-component systems (Perego & Hoch, 2002) drive the expression of metabolic genes in response to the presence of the nutrient in the medium.

CitM is the predominant citrate uptake system in *B. subtilis* under aerobic conditions (Warner & Lolkema, 2002). Previous studies showed that the sensor kinase CitS coded in the *citST* operon detects citrate in the external medium and transfers a signal to CitT, the response regulator. Phosphorylated CitT specifically binds to two sequences located in the region between −62 and −113 nucleotides upstream of *citM* transcriptional start point to activate transcription of the Mg^{2+}-citrate transporter (Yamamoto *et al.*, 2000). CitM is a secondary transporter that catalyzes proton motive force driven transport of citrate when it is complexed to Mg^{2+}, but also to the divalent metal ions Zn^{2+}, Co^{2+} and Ni^{2+} (Krom *et al.*, 2000). Transcription of the *citM* gene is repressed in the presence of several carbon sources (glucose, glycerol, inositol) by carbon catabolite repression (CCR) and by the nitrogen source arginine by an unknown mechanism (Warner *et al.*, 2000, 2003).

The main component in CCR in *B. subtilis* and other Gram-positive bacteria of low GC content is the global regulator CcpA (*C*atabolite *c*ontrol *p*rotein *A*) (Henkin, 1996; Stülke & Hillen, 2000). CcpA interacts with its cognate operator site (Catabolite Response Element, *cre* site; Kim & Chambliss, 1997) where it may act both as a repressor or an activator of transcription (Henkin, 1996). Binding of CcpA to *cre* sites is driven by complex formation with the phosphorylated forms of the coeffectors Hpr or Crh (Stülke & Hillen, 2000; Schumacher *et al.*, 2004). HPr and Crh are phosphorylated by HPr kinase at the expense of ATP in response to the accumulation of glycolytic intermediates such as fructose-1,6-bisphosphate following the uptake of glucose from the medium and to other metabolic signals (Galinier *et al.*, 1998; Jault *et al.*, 2000).

A *cre* sequence very close to the consensus sequence (Miwa *et al*., 2000) is found in the *citST* locus that codes for the two-component system responsible for the induction of the *citM* gene. This sequence is positioned in the *citS* gene 289 bp upstream from the *citT* translation initiation codon. In this report, we demonstrate that this *cre* site is functional in the repression of expression of the two-component system CitST by CCR, suggesting an additional mechanism of the control of expression of the Mg^{2+}-citrate transporter.

Materials and methods

Bacterial strains medium and growth conditions

Bacillus subtilis 168 derived strains used in this study are listed in Table 1. Cultures of *B. subtilis* strains were grown at 37 °C under continuous shaking at 250 r.p.m. in Luria–Bertani (LB) medium (Sambrook *et al*., 1989) or in minimal salts medium (MSM) containing 0.05% yeast extract (Warner & Lolkema, 2002). Antibiotics were used at the following concentrations: 5 μg chloramphenicol mL^{-1}; 20 μg kanamycin mL^{-1} and 100 μg spectinomycin mL^{-1}.

Construction of P*citST-lacZ* fusion series

The methods described by Sambrook *et al*. (1989) were used for manipulation of recombinant DNA. The series of integration vectors pEST was constructed by cloning different PCR fragments of the *citST* locus indicated in the Fig. 1 into the multiple cloning site of pJM116 (Dartois *et al*., 1996). Each region was obtained by PCR using the oligonucleotides indicated in Fig. 1 and Table S1 (supplementary data). The DNA sequence of the different fragments cloned in pJM116 plasmid was determined with automated DNA sequencing instrumentation at the University of Maine DNA sequencing Facility. *Bacillus subtilis* wild-type and mutant strains were transformed (Warner *et al*., 2000) with pEST vectors (as indicated in the third column of Tables 1 and S1, supplementary data) to yield the EST series of strains listed in Table 1. Successful integration into the *amyE* locus by homologous recombination was confirmed as described (Warner *et al*., 2000).

Table 1. *Bacillus subtilis* strains used in this study

Strain*	Genotype†	Source‡
168	*trpC2*	Warner *et al*. (2000)
QB5407	*trpC2 ccpA*::Tn917 *spc*	Faires *et al*. (1999)
QB7102	*trpC2 ptsH1 crh*::*aphA3*	Galinier *et al*. (1998)
EST410	*trpC2 amyE*::(P*citS-lacZ cat*)	pEST41 → 168
EST600	*trpC2 amyE*::(P*citS*$_i$ *-lacZ cat*)	pEST60 → 168
EST880	*trpC2 amyE*::(P*citST-lacZ cat*)	pEST88 → 168
EST882	*trpC2 ccpA*::Tn917 *spc amyE*::(P*citST-lacZ cat*)	pEST88 → QB5407
EST883	*trpC2 ptsH1 crh*::*aphA3 amyE*::(P*citST-lacZ cat*)	pEST88 → QB7102
EST920	*trpC2 amyE*::(*cre citT-lacZ cat)*	pEST92 → 168
EST940	*trpC2 amyE*::(P*citS* (−58,+52)-*lacZ cat*)	pEST94 → 168
EST960	*trpC2 amyE*::(P*citS* (+1,+52)-*lacZ cat*)	pEST96 → 168
EST980	*trpC2 amyE*::(P*citS* (−655, −36)-*lacZ cat*)	pEST98 → 168
EST100	*trpC2 amyE*::(P*citS* (+1,+1355)-*lacZ cat*)	pEST100 → 168

*All strains are derived from *B. subtilis* 168.

†Tn*917 spc*, Tn*917* derivative conferring resistance to spectinomycin; *aphA3*, *Enterococcus faecalis* kanamycin resistance gene; *cat*, pC194 chloramphenicol acetyltransferase gene.

‡The third column gives the plasmid and host strain for strains constructed during this study.

β-Galactosidase assay

β-Galactosidase activity was determined at 28 °C by a modification of the method of Miller (1972) using ONPG as the substrate. Cell extracts were obtained by treatment with lysosyme and Triton X-100 at final concentrations of 0.1 mg mL^{-1} and 0.1%, respectively. We present one representative experiment of at least three independent experiments. Standard deviation was calculated for duplicate measures of the same experiment and it was lower than the 10% of the value in each point of the graphics.

RNA isolation and analysis

Total RNA was isolated by a method described previously (Martin *et al*., 2004). Total RNA concentration was determined by UV spectrometry and by gel quantification with Gel Doc 1000 (Bio-Rad). For Slot blot analysis, different quantities of total RNA isolated from *B. subtilis* 168 strain and from an isogenic *ccpA* mutant strain (QB5407) were used. Transfer of nucleic acids to nitrocellulose membranes and hybridization with radioactive probes were performed as described previously (Martin *et al*., 2004). The double-stranded probe hybridizing with *citST* (Probe I) was labelled by incorporation of [α-^{32}P] dATP using the commercial kit Prime-a-gen labeling System (Promega; Fig. 2). Primer extension analysis was performed as previously described (Martin *et al*., 2004). The assays were scanned in an Amersham Biosciences Phosphorimager.

Purification of CcpA and Gel mobility shift assays

Escherichia coli M15 (pREP4) transformed with pQE30-CcpA (Moir-Blais *et al*., 2001) was grown in LB until $OD_{600\,nm} = 0.7$ and induced by addition of 2 mM IPTG during 4 h. Cells were harvested and disrupted. The His_6-CcpA in the supernatant was purified by nickel-NTA column chromatography (Novagen) and then dialyzed and

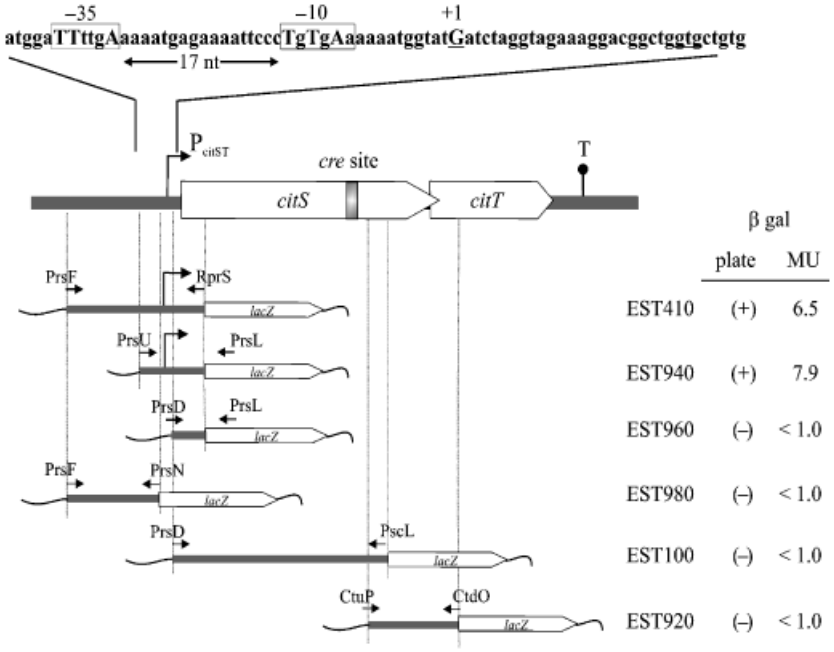

Fig. 1. Schematic representation of the *citST* locus and *lacZ* transcriptional fusions. The sequence shown in the top of the figure represents the results of primer extension assay with the putative −35 and −10 boxes boxed (nucleotides that match the consensus sequence recognized by the σ^A transcriptional factor were indicated in uppercase characters), the transcriptional initiation site was marked (+1) and the translation initiation site was underlined. On the right, the activity of the *lacZ* fusions is indicated on X-Gal plates (+ and − represent blue and white colonies, respectively) and in the β-galactosidase activity assay expressed in Miller units (MU).

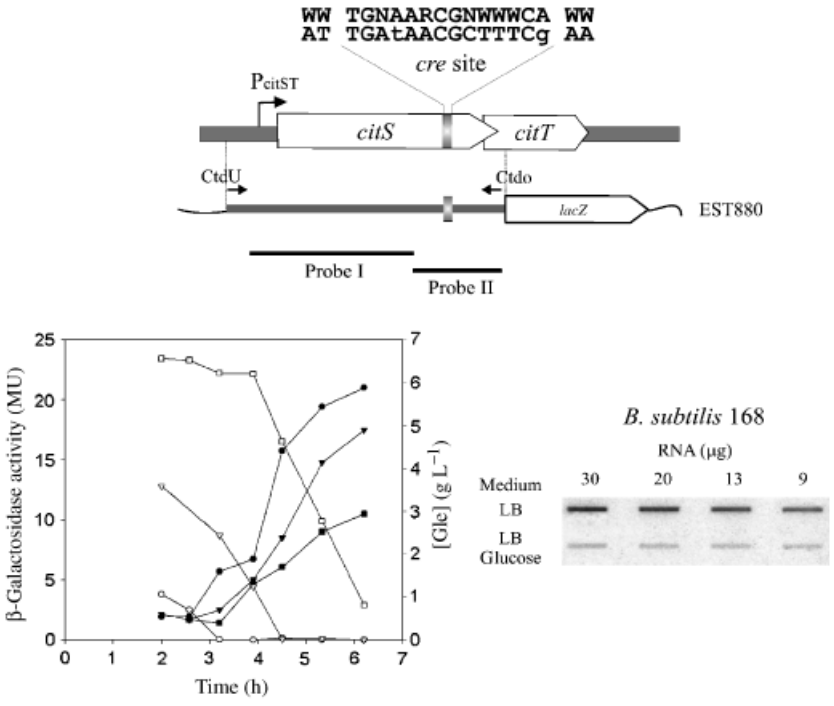

Fig. 2. Top. Diagram of the *citST* operon with the *cre* site in the *citS* gene. Nucleotides that do not match the consensus sequence for *cre* sites are indicated in lowercase letters (W represents A or T, N any nucleotide, and R represents A or G). The transcriptional fusion corresponding to strain EST880 is represented below. Bottom, left. β-galactosidase activity (closed symbols) and glucose concentration in the medium (open symbols) during growth of *Bacillus subtilis* EST880 harboring the P*citST-lacZ* fusion in LB-Citrate (LBC) medium supplied with different glucose concentrations: 0.2% w/v (●,○); 0.4% w/v (▼, open wedge); 0.8% w/v (■,□). Bottom, right. Slot-blot assay of total mRNA isolated from wild-type *B. subtilis* 168 grown in LB and LB plus glucose as indicated and using the sequence schematically indicated as Probe I in the top figure as probe.

stored at −80 °C for further studies as described in the supplementary data. Protein concentration was determined by Bradford method (Sambrook *et al*., 1989). A DNA fragment including *citS cre* site (Probe II, Fig. 2) was prepared by PCR using primers CtuP and CtdO (Table S1, supplementary data). This 590 bp fragment was labelled by incorporation of [α-^{32}P] dATP in a PCR amplification. Binding of CcpA to the DNA fragment was carried out in a 15 μL reaction mix containing 10 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 50 μg BSA mL^{-1}, 0.05% v/v Nonidet and 5% v/v glycerol. After 15 min of incubation at 37 °C, the samples were applied to a 5% w/v

polyacrylamide gel, which had been prerun for 1 h in 45 mM Tris-borate pH 8, 1 mM EDTA. Band patterns were visualized in an Amersham Biosciences Phosphorimager.

Analytical methods

Glucose concentration in the culture medium was determined enzymatically with a glucose oxidase-peroxidase based system following the protocol provided by the supplier (Wiener Labs test kit).

Results

Identification of the *citST* promoter region

A series of transcriptional fusions to the *lacZ* reporter gene was constructed containing different fragments of the *citST* region (Fig. 1, and Table S1, supplementary data). The constructs were integrated at the *amyE* locus on the chromosome of *B. subtilis* strain 168. The resulting strains were plated on β-galactosidase indicator plates containing the chromogenic substrate X-Gal and the β-galactosidase activity of the cells grown on LB liquid medium was measured at the end of the exponential growth phase after 5 h of growth (Fig. 1). Fused to *lacZ* in strain EST410 is a 707 bp DNA fragment which corresponds to a region starting upstream of the *citS* gene and extending 52 bp into the gene (P*citS-lacZ*). The fragment includes the putative promoter region (Fig. 1). The strain showed a positive phenotype on indicator plates and quantitative measurement of β-galactosidase activity revealed a 10 times higher activity than observed in strain EST600, which contains the same 707 bp fragment but in opposite orientation (P$citS_i$-*lacZ*). Reducing the size of the fragment in the fusion constructs at the 5′ end by some 600 bp (strain EST940) gave similar results, but deletion of an additional 50 bp (EST960) including the putative promoter resulted in a strain that was devoid of β-galactosidase activity both on plates and in the activity assay. Deleting the putative promoter by reducing the size of the 707 bp fragment in EST410 by some 80 bp at the 3′ end yielding strain EST980 similarly resulted in a negative phenotype. These results pinpoint the promoter of the *citST* operon between nucleotides −58 to +1 relative to the *citS* gene. The *citST* transcriptional start point was determined by primer extension using total RNA isolated from *B. subtilis* strain 168 as template. Primer CstW (Table S1, supplementary data) resulted in a weak signal corresponding to a 103 bp product consistent with the transcriptional start point indicated in Fig. 1 (data not shown). The Neural Network Promoter Predictor (NNPP, version 2.2) which was specifically designed for *B. subtilis* (Reese, 2001) identified the same guanine residue located 27 bp upstream of the *citS* start codon (GTG) as the transcriptional initiation site (score 0.99). Boxes −10 (TTTTGA) and −35 (TGTGAA) are separated by 17 bp and are located in positions −10 through −15 and −33 through −38, respectively. A comparison with the consensus sequence recognized by the σ^A factor shows a 50% homology with respect to the putative boxes proposed. Secondary structure prediction by Mfold (version 3.1; Zuker, 2003) revealed a stem–loop conformation in the RNA sequence corresponding to the region downstream of the *citT* gene (ΔG° of $-21.2\,kcal\,mol^{-1}$), which could act as a ρ independent transcriptional terminator of the *citST* operon.

Strains EST920 and EST100 were constructed to identify putative alternative promoters of *citT* expression. The former contains a 590 bp DNA fragment that corresponds to the *citT* 5′ region fused to the *lacZ* gene; the latter contains nucleotides +1 to +1355 of the *citS* gene. The results obtained for both strains were the same as observed for the control strain EST600 indicating that no second promoter is present in the region upstream of the *citT* gene. In agreement, no product was obtained when total RNA isolated from wild-type 168 strain was assayed by primer extension with primers CtdO or PecT (Table S1, supplementary data) targeted at 113 and 12 bp relative to the *citT* translational start site, respectively. All these results demonstrate that *citS* and *citT* form part of a bicistronic operon.

CcpA-dependent repression of expression of the *citST* operon by glucose

The transcriptional expression of β-galactosidase from the *citST* promoter in the absence of the *cre* site in front of the *citT* gene (strain EST410) was analyzed in minimal medium and complex LB medium to which the effectors of the expression of the *citM* gene, the target of the CitST two-component system, were added. Expression of the *citM* gene is induced by citrate and repressed by glucose. Neither in minimal or complex medium, the presence of citrate or glucose or both had a significant effect on the expression profile of β-galactosidase (see Table S2, supplementary data). In contrast, β-galactosidase activity of strain EST880 was significantly lower when grown in the presence of glucose. EST880 carries integrated in the *amyE* site transcriptional fusion P*citST-lacZ* that contains a DNA fragment including the promoter region and the *cre* site in front of the *citT* gene (Fig. 2). The levels of expression of β-galactosidase in strain EST880 were about three times lower at the end of the exponential growth phase when 0.4% of glucose was included in minimal growth medium (Fig. S1, supplementary data). As shown in the Fig. 2, the repression by glucose was confirmed when strain EST880 was grown in rich medium (LB) in the presence of increasing initial glucose concentrations. At increasing concentrations, the rise in β-galactosidase levels in the cells shifted to later times in the growth curve, correlating with the decrease of the glucose

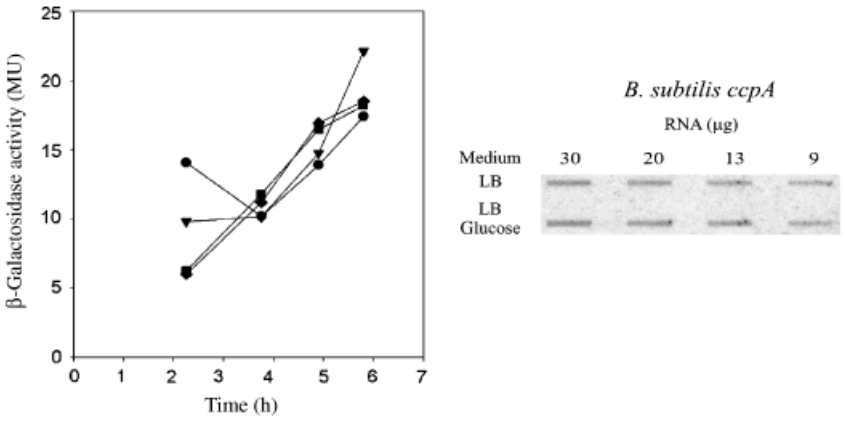

Fig. 3. Left. β-galactosidase activity of strains EST882 (*ccpA* mutant; ■,◆) and EST883 (*crh ptsH*1 mutant; ▼,●) harbouring the P*citST-lacZ* fusion. The strains were grown in LB-Citrate (■,▼) and LBC plus 0.4% w/v glucose (◆,●). Right. Slot-blot assay of total mRNA isolated from the *ccpA* mutant strain grown in LB and LB plus glucose as indicated and using the sequence schematically indicated as Probe I in the top of Fig. 2 as probe.

concentration in the medium (Fig. 2). CcpA is a major player in CCR in Gram-positive bacteria that binds to *cre* sites to exert its regulatory effect. The involvement of CcpA in the repression in strain EST880 was demonstrated by integration of the transcriptional fusion in the *amyE* locus of a strain deficient in CcpA yielding strain EST882. In this strain, the presence of glucose in the medium did not repress the expression of the transcriptional fusion (Fig. 3). In *B. subtilis*, CcpA binds to the *cre* site in complex with either HPr or Crh. To test whether these factors would affect the regulation of *citST* expression, we introduced the P*citST-lacZ* fusion in a deficient strain in HPr and Crh (*ptsH1 crh*) yielding strain EST883. Similarly, as observed for strain EST882, in strain EST883, the expression level of β-galactosidase was not affected by the presence of glucose in the medium (Fig. 3).

The levels of *citST* mRNA in the wild-type strain 168 and an otherwise isogenic CcpA deficient strain (QB5407) grown in the presence and absence of glucose were analyzed by a Slot blot assay (Figs 2 and 3, respectively). Total RNA was isolated and different quantities were hybridized with probe I (see Fig. 2). It follows that the level of the *citST* messenger was significantly lower in strain 168 when the cells were grown in the presence of glucose suggesting regulation at the transcriptional level. In contrast, no difference was observed in the mRNA levels in strain QB5407 (*ccpA* mutant) when grown in the presence or absence of glucose, clearly showing the involvement of the CcpA protein in the regulation.

CcpA binds to the *cre* site located in the *citST* locus

Direct binding of CcpA to the *cre* site found in the *citS*-coding region was demonstrated by a gel mobility shift assay. *Bacillus subtilis* CcpA was over expressed and purified as described in the Methods section. The electrophoretic mobility of a radioactively labeled DNA fragment (590 bp, probe II in Fig. 2) corresponding to nucleotides −454 to +136 relative to *citT* translational start site (Fig. 4), which included the *cre* site, was specifically retarded by the purified CcpA protein (Fig. 4). At the lowest protein concentration, almost all labeled DNA was free but the addition of as little as 15 ng of CcpA was sufficient to induce complex formation. A 174 bp long DNA fragment from the *B. subtilis acpA* promoter region was used as a control. The mobility of the fragment was insensitive to the presence of CcpA up to 40 ng demonstrating the specificity of the binding (data not shown). In addition, a 20-fold excess of the unlabeled 590 bp fragment of the *citST* region prevented binding of the labeled fragment (data not shown). This observation is in good agreement with our previous results that showed specific binding of CcpA to the *citS cre* site.

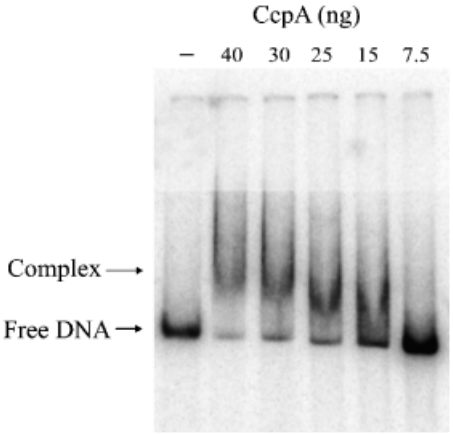

Fig. 4. *In vitro* binding of CcpA to the *citS cre* site. Different amounts of purified *Bacillus subtilis* CcpA protein were incubated with a DNA fragment corresponding to the region indicated as Probe II in the top of Fig. 2. Arrows indicate the positions of the retarded complex and the free DNA fragment.

Discussion

The data presented in this paper indicate that in *B. subtilis* expression of the genes *citS* and *citT* that code for a

two-component system involved in the induction of the Mg^{2+}-citrate transporter CitM is regulated by CCR. While a *lacZ* transcriptional fusion to the *citST* promoter region showed the same pattern of expression in the presence or absence of glucose, a fusion construct including the putative *cre* site in the *citS* gene was down regulated in response to the availability of glucose in the medium. Measurement of the *citST* mRNA levels showed a decreased level in the presence of glucose demonstrating regulation of transcription. The involvement of the CCR system was demonstrated by the lack of repression by glucose when the P*citST-lacZ* transcriptional fusion including the *cre* site was introduced in *B. subtilis* strains deficient in the CCR transcriptional regulator CcpA or the coeffectors of CcpA, HPr and Crh. Finally, the functionality of the *cre* site in the *citS* gene was demonstrated by binding of the CcpA protein to a DNA fragment containing the cognate site for this regulator.

The present findings suggest a complex regulation of expression of the Mg^{2+}-citrate transporter CitM in *B. subtilis* (Fig. 5). The global regulator CcpA directly regulates transcription of the *citM* gene through the *cre* site in the *citM* promoter region and, indirectly, by regulating the expression of the two-component system responsible for the induction of *citM* transcription. It follows that metabolic conditions that induce CcpA binding to cre sites repress *citM* transcription and, at the same time, could reduce *citM* induction, which would result in an amplified down regulation of expression of the CitM protein.

The sophisticated control of expression of the citrate transporter CitM may serve two purposes; one, protection against toxic divalent metal ions and, two, fine tuning of transcriptional control of TCA cycle enzymes. CitM transports citrate into the cell when complexed to a well defined set of divalent metal ions, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} (Krom *et al.*, 2000). The metal ion specificity of the complex renders *B. subtilis* extremely sensitive to the toxic heavy metal ions Zn^{2+}, Ni^{2+}, and Co^{2+} when citrate is present in the medium and CitM in the membrane (Krom *et al.*, 2002). Then, reducing the level of expression to zero levels is essential for survival. On the other hand, uptake of citrate from the medium by CitM will interfere directly with the regulation of expression of the Krebs cycle enzymes. Most of these Krebs cycle enzymes are greatly repressed in the presence of a readily metabolized carbon source, such as glucose (Kim *et al.*, 2002; Sonenshein, 2002; Commichau *et al.*, 2006; see Fig. 5). The first two steps of the TCA cycle are regulated by CcpA and the specific transcriptional regulator CcpC. CcpC, a member of the LysR family of

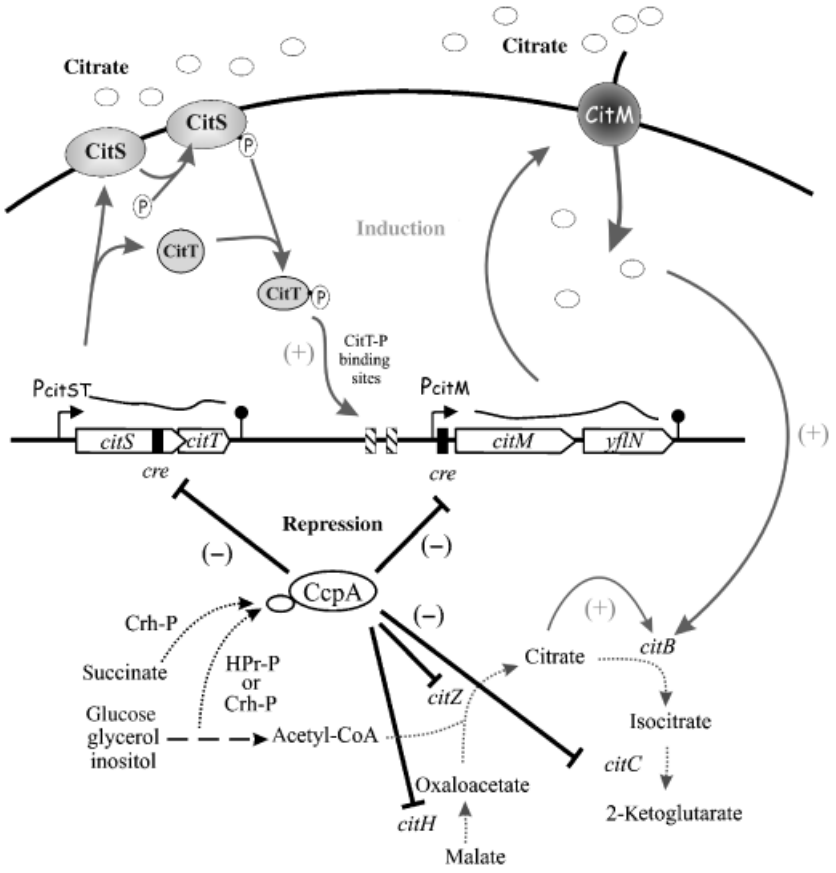

Fig. 5. Regulation of expression of citrate metabolism in *Bacillus subtilis*. The induced state of the system in the presence of citrate is indicated in gray. Transcriptional repression mediated by CcpA in the presence of rapidly degraded carbon sources is indicated in black (see text for explanation).

transcriptional regulators, straightforwardly repress the *citB* gene (Aconitate hydratase). CcpA was shown to directly repress the expression of *citZ* (Citrate synthase II), whereas it plays an indirect role in the regulation of *citB* expression (Commichau *et al.*, 2006). It was demonstrated that citrate is the effector of CcpC and that CcpC is not able to repress *citB* transcription in the presence of citrate (Kim *et al.*, 2002). In the absence of externally provided citrate, the intracellular pool of this intermediate is synthesized exclusively by CitZ from acetyl-CoA and oxaloacetate. Clearly, influx of external citrate from the medium mediated by CitM will interfere with this subtle regulatory system and may require strict control of CitM expression.

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References

Commichau FM, Forchhammer K & Stülke J (2006) Regulatory links between carbon and nitrogen metabolism. *Curr Opin Microbiol* **9**: 1–6.

Dartois V, Djavakhishvili T & Hoch JA (1996) Identification of a membrane protein involved in activation of the KinB pathway to sporulation in *Bacillus subtilis*. *J Bacteriol* **178**: 1178–1186.

Faires N, Tobisch S, Bachem S, Martin-Verstraete I, Hecker M & Stulke J (1999) The catabolite control protein CcpA controls ammonium assimilation in *Bacillus subtilis*. *J Mol Microbiol Biotechnol* **1**: 141–148.

Galinier A, Kravanja M, Engelmann R, Hengstenberg W, Kilhoffer MC, Deutscher J & Haiech J (1998) New protein kinase and protein phosphatase families mediate signal transduction in bacterial catabolite repression. *Proc Natl Acad Sci USA* **95**: 1823–1828.

Henkin TM (1996) The role of CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. *FEMS Microbiol Lett* **135**: 9–15.

Jault JM, Fieulaine S, Nessler S, Gonzalo P, Di Pietro A, Deutscher J & Galinier A (2000) The HPr Kinase from *Bacillus subtilis* is a homo-oligomeric enzyme which exhibits strong positive cooperativity for nucleotide and fructose 1,6-bisphosphate binding. *J Biol Chem* **275**: 1773–1780.

Kim HJ & Chambliss GH (1997) Contacts between *Bacillus subtilis* catabolite regulatory protein CcpA and *amyO* target site. *Nucleic Acids Res* **25**: 3490–3496.

Kim HJ, Roux A & Sonenshein AL (2002) Direct and indirect roles of CcpA in regulation of *Bacillus subtilis* Krebs cycle genes. *Mol Microbiol* **45**: 179–190.

Krom BP, Warner JB, Konings WN & Lolkema JS (2000) Complementary metal ion specificity of the metal-citrate transporters CitM and CitH of *Bacillus subtilis*. *J Bacteriol* **182**: 6374–6381.

Krom BP, Huttinga H, Warner JB & Lolkema JS (2002) Impact of the Mg^{+2}-citrate transporter CitM on heavy metal toxicity in *Bacillus subtilis*. *Arch Microbiol* **178**: 370–375.

Martin MG, Sender PD, Peiru S, de Mendoza D & Magni C (2004) Acid-inducible transcription of the operon encoding the citrate lyase complex of *Lactococcus lactis* Biovar diacetylactis CRL264. *J Bacteriol* **186**: 5649–5660.

Miller JH (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Miwa Y, Nakata A, Ogiwara A, Yamamoto M & Fujita Y (2000) Evaluation and characterization of catabolite-responsive elements (*cre*) of *Bacillus subtilis*. *Nucleic Acids Res* **28**: 1206–1210.

Moir-Blais TR, Grundy FJ & Henkin TM (2001) Transcriptional activation of the *Bacillus subtilis ackA* promoter requires sequences upstream of the CcpA binding site. *J Bacteriol* **183**: 2389–2393.

Perego M & Hoch J (2002) Two-component systems, phosphorelays and regulation of their activities. *Bacillus subtilis and its Closest Relatives: From the Genes to Cells* (Sonenshein AL, Hoch JA & Losick R, eds), pp. 473–482. American Society for Microbiology Press, Washington, DC.

Reese MG (2001) Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Comput Chem* **26**: 51–56.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schumacher M, Allen G, Diel M, Seidel G, Hillen W & Brennan R (2004) Structural basis for allosteric control of the transcription regulator CcpA by the phosphoprotein HPr-Ser46-P. *Cell* **118**: 731–741.

Sonenshein AL (2002) The Krebs citric acid cycle. *Bacillus subtilis and its Closest Relatives: From the Genes to Cells* (Sonenshein AL, Hoch JA & Losick R, eds), pp. 151–162. American Society for Microbiology Press, Washington, DC.

Stülke J & Hillen W (2000) Regulation of carbon catabolism in *Bacillus* species. *Annu Rev Microbiol* **54**: 849–880.

Warner JB & Lolkema JS (2002) Growth of *Bacillus subtilis* on citrate and isocitrate is supported by the Mg^{2+}-citrate transporter CitM. *Microbiology* **148**: 3413–3421.

Warner JB, Krom BP, Magni C, Konings WN & Lolkema JS (2000) Catabolite repression and induction of the Mg^{+2}-citrate transporter CitM of *Bacillus subtilis*. *J Bacteriol* **182**: 6099–6105.

Warner JB, Magni C & Lolkema JS (2003) CcpA-independent regulation expression of the Mg^{2+}-citrate transporter gene

citM by arginine metabolism in *Bacillus subtilis*. *J Bacteriol* **185**: 854–859.

Yamamoto H, Murata M & Sekiguchi J (2000) The CitST two-component system regulates the expression of the Mg-citrate transporter in *Bacillus subtilis*. *Mol Microbiol* **37**: 898–912.

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**: 3406–3415.

Supplementary material

The following supplementary material is available for this article online:

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Appendix S1.

Table S1. PCR fragments and oligonucleotides employed in the construction of *lacz* transcriptional fusions.

Table S2. β-Galactosidase activity of *B. subtilis* strain EST410 grown in minimal salt medium (MSM) and complex medium (LB).

Fig S1. β-galactosidase activity of *citST-lacZ* fusion (EST880).

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